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Patentanmeldung Nr. Patent application No. Demande de brevet n°

02021602.4

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk

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(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Biological process for producing l-ascorbic acid

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Case 21409

Biological Process for Producing L-Ascorbic acid

The present invention relates to the use of Enzyme B of *Gluconobacter oxydans* DSM 4025 as disclosed in EP 832,974 in a process for producing L-ascorbic acid.

Feasibility studies on the biotechnological synthesis of L-ascorbic acid (AsA) were performed for many years since the "Reichstein method" was established in 1934. The microorganisms *Gluconobacter oxydans* DSM 4025, *Escherichia coli* carrying the D-arabinono-1,4-lactone oxidase gene of *Saccharomyces cerevisiae*, *Candida albicans* and *Saccharomyces cerevisiae* oxidize L-galactono-1,4-lactone to AsA. *Saccharomyces cerevisiae* and *Candida albicans* possess a D-arabinose dehydrogenase catalyzing the production of D-arabinono-1,4-lactone and L-galactono-1,4-lactone from D-arabinose and L-galactose, respectively. However, there were no reports describing the possibility of biological AsA production from another L-hexose as intermediate, that is, L-idose, L-gulose, and L-talose, with a configuration corresponding to that of AsA (C4 and C5 positions).

The present invention provides the use of Enzyme B of *G. oxydans* DSM 4025, as disclosed in EP 832,974, in a process for producing L-ascorbic acid from L-gulose, L-galactose, L-idose or L-talose, or from L-gulono-1,4-lactone (and its acid form, L-gulonic acid) and from L-galactono-1,4-lactone (and its acid form, L-galactonic acid).

The present invention also provides the use of Enzyme B of *G. oxydans* DSM 4025, as disclosed in EP 832,974, in a process for producing L-gulono-1,4-lactone or L-galactono-1,4-lactone, or their acid forms L-gulonic acid or L-galactonic acid from L-gulose or L-galactose, respectively.

L-Hexoses like L-gulose, L-galactose, L-idose, and L-talose are rare sugars which are basically produced by chemical methods and are commercially high-cost compounds. How-

ever, biological preparations for L-gulose and L-galactose have been recently reported. L-Gulose production from D-sorbitol by Enzyme A of *G. oxydans* DSM 4025 was reported in EP 832,974. L-Gulose production from L-sorbose by L-ribose isomerase was disclosed in US 6,037,153. L-Galactose production from L-sorbose is reported by Izumori et al. (2001 Annual Meeting of the Society for Bioscience and Bioengineering, Japan). In this process they combined two enzymatic processes consisting of "L-sorbose to L-tagatose" reaction with L-tagatose epimerase of *Pseudomonas cichorii* ST-24 strain (US 5,811,271) and "L-tagatose to L-galactose" reaction with D-arabinose isomerase of *Bacillus stearothermophilus* 14a strain. L-Gulono-1,4-lactone may be prepared from D-glucose.

In another aspect the present invention provides a process for

(a) producing

(i) L-ascorbic acid from L-gulose or L-galactose by an enzyme which comprises contacting L-gulose or L-galactose with an enzyme having an amino acid sequence of SEQ ID NO:2 or an amino acid sequence that is 90% identical thereto, or a portion thereof, with the activity to produce L-ascorbic acid from L-gulose and L-galactose or a functional equivalent thereof, in a reaction mixture,

(ii) L-gulono-1,4-lactone from L-gulose, or L-galactono-1,4-lactone from L-galactose by an enzyme which comprises contacting L-gulose or L-galactose with an enzyme having an amino acid sequence of SEQ ID NO:2 or an amino acid sequence that is 90% identical thereto, or a portion thereof, with the activity to produce L-gulono-1,4-lactone from L-gulose and L-galactono-1,4-lactone from L-galactose, or a functional equivalent thereof, in a reaction mixture, or

(iii) L-ascorbic acid from L-gulono-1,4-lactone or from L-galactono-1,4-lactone by an enzyme which comprises contacting L-gulono-1,4-lactone or from L-galactono-1,4-lactone with the enzyme having an amino acid sequence of SEQ IDNO:2 or an amino acid sequence that is 90% identical thereto, or a portion thereof, with the activity to produce L-ascorbic acid from L-gulono-1,4-lactone and from L-galactono-1,4-lactone, or a functional equivalent thereof, in a reaction mixture,

and

(b) isolating L-ascorbic acid, L-gulono-1,4-lactone or L-galactono-1,4-lactone from the reaction mixture.

In the present invention, a functional equivalent of the enzyme can be made either by chemical peptide synthesis known in the art or by recombinant means on the basis of the DNA sequences as disclosed herein by methods known in the state of the art. Amino acid exchanges in proteins and peptides which do not generally alter the activity of such mole-

cules are known in the state of the art. The most commonly occurring exchanges are: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly as well as these in reverse.

Furthermore such functional equivalent of the enzyme includes an amino acid sequence encoded by a DNA sequence of SEQIDN:1 as disclosed e.g. in the sequence listing as well as the complementary strand, or those which include the sequences, DNA sequences which hybridize under standard conditions with such sequences or fragments thereof and DNA sequences, which because of the degeneration of the genetic code, do not hybridize under standard conditions with such sequences but which code for polypeptides having exactly the same amino acid sequence, wherein the functional equivalent has the enzymatic activity of producing
(i) L-ascorbic acid from L-gulose and from L-galactose,
(ii) L-gulono-1,4-lactone (and its acid form, L-gulonic acid) from L-gulose and L-galactono-1,4-lactone (and its acid form, L-galactonic acid) from L-galactose, or
(iii) L-ascorbic acid from L-gulono-1,4-lactone (and its acid form, L-gulonic acid) and from L-galactono-1,4-lactone (and its acid form, L-galactonic acid).

"Standard conditions" for hybridization mean in this context the conditions which are generally used by a man skilled in the art to detect specific hybridization signals, or preferably so called stringent hybridization and non-stringent washing conditions or more preferably so called stringent hybridization and stringent washing conditions a man skilled in the art is familiar with. Furthermore, DNA sequences which can be made by the polymerase chain reaction by using primers designed on the basis of the DNA sequences disclosed herein by methods known in the art are also an object of the present invention. It is understood that the DNA sequences of the present invention can also be made synthetically as described, e.g. in EP 747,483.

A mutant of the gene can be prepared by treating the gene or a microorganism carrying the gene with a mutagen such as ultraviolet irradiation, X-ray irradiation, γ-ray irradiation or contact with a nitrous acid, N-methyl-N'-nitro-N-nitrosoguanidine (NTG), or other suitable mutagens, or isolating a colony or clone occurring by spontaneous mutation or by standard methods of *in vitro* mutagenesis known in the art. Many of these methods have been described in various publications.

As used herein, a "mutant" is any gene that encodes a non-native polynucleotide sequence or a polynucleotide sequence that has been altered from its native form (such as, e.g., by rearrangement or deletion or substitution of from 1-100, preferably 20-50, more

preferably less than 10 nucleotides). As noted above, such a non-native sequence may be obtained by random mutagenesis, chemical mutagenesis, spontaneous mutation, UV-irradiation, PCR-prone error generation, site-directed mutagenesis, and the like. Preferably, the mutation results in texpressing polypeptide having the increased production or improved activity compared to a non-mutant parental polypeptide using the assay procedures set forth in the Examples. Methods for generating, screening for, and identifying such mutant cells are well known in the art.

A specific and preferred *G. oxydans* strain as a donor of a DNA sequence encoding a polypeptide has been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) in Göttingen (Germany) under DSM No. 4025. A biologically and/or taxonomically homogeneous culture of a microorganism having the identifying characteristics *G. oxydans* DSM 4025 can also be a donor of the DNA sequence.

A subculture of *G. oxydans* DSM 4025 has been deposited in the Agency of Industrial Science and Technology, Fermentation Research Institute, Japan, under the deposit No.: FERM BP-3812. EP 278,477 discloses the characteristics of this strain.

By using the information of the so determined nucleotide sequence (in consideration of the codon usage) a gene encoding evolutionally divergent enzyme having the activity of producing (i) L-ascorbic acid from L-gulose and L-galactose, (ii) L-gulono-1,4-lactone (and its acid form, L-gulonic acid) from L-gulose and L-galactono-1,4-lactone (and its acid form, L-galactonic acid) from L-galactose, or (iii) L-ascorbic acid from L-gulono-1,4-lactone (and its acid form, L-gulonic acid) and from L-galactono-1,4-lactone (and its acid form, L-galactonic acid).AsA forming-activity from L-gulose or L-galactose, can be isolated from a different organism by colony- or Southern-hybridization with a probe synthesized according to the amino acid sequence deduced from said nucleotide sequence or by the polymerase chain reaction with primers also synthesized according to said information, if necessary.

Furthermore, a preferred host microorganism for constructing a recombinant microorganism carrying Enzyme B gene of *G. oxydans* DSM 4025 and its functional equivalent or mutant defined above may be *Escherichia coli, Pseudomonas putida* and *G. oxydans* DSM 4025 and their biologically and/or taxonomically homogeneous culture or mutant.

To construct a recombinant microorganism carrying the Enzyme B gene and its functional equivalent or mutant on a recombinant expression vector or on a chromosomal DNA of a host microorganism, various gene transfer methods including transformation, transduc-

tion, conjugal mating, and electroporation can be used. The method for constructing a recombinant organism may be selected from the methods well-known in the field of molecular biology. Usual transformation systems can be used for *Escherichia coli*, and *Pseudomonas*. A transduction system can also be used for *Escherichia coli*. Conjugal mating systems can be widely used in Gram-positive and Gram-negative bacteria including *E. coli*, *Pseudomonas putida* and *G. oxydans*. The conjugation can occur in liquid media or on a solid surface. The preferred recipient is selected from *E. coli*, *Pseudomoas putida* and *G. oxydans* which can produce active Enzyme B with a suitable recombinant expression vector. To the recipient for conjugal mating, a selective marker is usually added; for example, resistance against nalidixic acid or rifampicin is usually selected.

The microorganisms provided in the present invention may be cultured in an aqueous medium supplemented with appropriate nutrients under aerobic conditions. The cultivation may be conducted at a pH between about 1.0 and 9.0, preferably between about 2.0 and 8.0. While the cultivation period varies depending upon pH, temperature and nutrient medium used, usually 2 to 5 days will bring about favorable results. A preferred temperature range for carrying out the cultivation is from about 13°C to 45°C preferably from about 18°C to 42°C.

It is usually required that the culture medium contains such nutrients as assimilable carbon sources, digestible nitrogen sources and inorganic substances, vitamins, trace elements and other growth promoting factors. Examples for assimilable carbon sources include glycerol, D-glucose, D-mannitol, D-fructose, D-arabitol, D-sorbitol and L-sorbose.

Various organic or inorganic substances may also be used as nitrogen sources, such as yeast extract, meat extract, peptone, casein, corn steep liquor, urea, amino acids, nitrates, ammonium salts and the like. As inorganic substances, magnesium sulfate, potassium phosphate, ferrous and ferric chlorides, calcium carbonate and the like may be used.

If there is no clear definition, L-ascorbic acid means that the substance exists either as a free acid form or as a salt form such as Na-salt, K-salt, or hemicalcium-salt. Moreover, concentration of L-ascorbic acid is described as the free acid form unless otherwise stated.

If there is no clear definition, L-gulono-1,4-lactone and L-galactono-1,4-lactone mean that the substances exist as their lactone forms and/or their acid forms, both of which exist in an equilibrium state under various physico-chemical conditions.

As used herein the phrase "standard conditions for hybridization" means conditions which are generally used by a person skilled in the art to detect specific hybridization signals, or preferably, so called stringent hybridization and non-stringent washing conditions, or more preferably, so called moderately stringent conditions, or even more preferably, so called stringent hybridization and stringent washing conditions which a person skilled in the art is familiar with.

For example, any combination of the following hybridization and wash conditions may be used, as appropriate:

High Stringency Hybridization: 6X SSC, 0.5% SDS, 100 µg/ml denatured salmon sperm DNA, 50% formamide, incubate overnight at 42°C with gentle rocking.

High Stringency Wash: 1 wash in 2X SSC, 0.5% SDS at room temperature for 15 minutes, followed by another wash in 0.1X SSC, 0.5% SDS at room temperature for 15 minutes.

Low Stringency Hybridization: 6X SSC, 0.5% SDS, 100 µg/ml denatured salmon sperm DNA, 50% formamide, incubate overnight at 37°C with gentle rocking.

Low Stringency Wash: 1 wash in 0.1X SSC, 0.5% SDS at room temperature for 15 minutes.

Moderately stringent conditions may be obtained by varying the temperature at which the hybridization reaction occurs and/or the wash conditions as set forth above.

The concentration of the substrates including L-aldose and L-aldonolactone in a reaction mixture can vary depending on other reaction conditions, but, in general, may be between 1 g/l and 300 g/l, preferably between 10 g/l and 200g/l.

The reaction can be conducted aerobically.

For the reaction, any forms of enzyme can be used; enzyme solution, immobilized enzyme, intact cell, and immobilized cell may be used.

After the reaction, L-ascorbic acid or L-aldonolactone may be recovered from the reaction mixture by the combination of various kinds of chromatography, for example, thin layer chromatography, adsorption chromatography, ion-exchange chromatography, gel filtration chromatography or high performance liquid chromatography. L-Aldonolactone as a reaction product can also be used as a substrate for a further reaction as it is in the reaction mixture of this invention without purification.

The following examples are provided to further illustrate the process of the present invention. These examples are illustrative only and are not intended to limit the scope of the invention in any way.

Example 1: Production of L-gulono-1,4-lactone/L-gulonic acid from L-gulose by *Escherichia coli* JM109 carrying the Enzyme B gene

Enzyme B gene was cloned and subcloned from pSS103R into a vector pTrcMalE to construct pTrcMalE-EnzB as described in EP 832,974. *Escherichia coli* JM109 carrying pTrcMalE-EnzB was grown on 2 ml of LB medium with 100 µg/ml of ampicillin at 30°C for 15 hours and 100 µl of the resulting broth was transferred into fresh LB medium with 100 µg/ml of ampicillin and incubated at 30°C for 4 hours. Then, IPTG was added to the culture at a final concentration of 0.2 mM and the culture was further cultivated at 30°C for 2 hours. The control cultivation of *E. coli* JM109 carrying pTrcMalE-EnzB without IPTG addition was also performed. *Escherichia coli* JM109 was used as the control strain in the same manner as described above, cultivation with or without addition of IPTG. The cells from 8 ml of the culture were collected by centrifugation and suspended in 1 ml of distilled water. The resulting cell suspension was used for the reaction with 400 µl of reaction mixture consisting of 250 µl of the cell suspension, 1% substrate, 0.3% NaCl, 1% $CaCO_3$, 1 µg/ml of PQQ and 1 mM PMS and the mixture was incubated at room temperature for 16 hr. The substrate used in this experiment was L-gulose. The amounts of L-gulono-1,4-lactone plus L-gulonic acid and AsA are summarized in Table 1.

Table 1

Strain	IPTG	HPLC	
		L-GuL+L-GuA (mM)	AsA (mg/L)
JM109 /pTrcMalE-EnzB	+	8.4	2.5
	-	4.6	10.5
JM109	+	nd	nd
	-	nd	nd

L-GuL: L-gulono-1,4-lactone; L-GuA: L-gulonic acid; nd: not detected

Example 2: Production of AsA from L-gulono-1,4-lactone/L-gulonic acid by *Escherichia coli* JM109 carrying the Enzyme B gene

The experiment was performed as described in Example 1, except of the substrate used which was L-gulono-1,4-lactone in this Example. The amount of AsA is summarized in Table 2.

Table 2

Strain	IPTG	HPLC
		AsA (mg/L)
JM109 /pTrcMalE-EnzB	+	1.4
	-	1.2
JM109	+	nd
	-	nd

nd: not detected

Example 3: Production of L-galactono-1,4-lactone/L-galactonic acid from L-galactose by *Escherichia coli* JM109 carrying the Enzyme B gene

The experiment was performed as described in Example 1, except of the substrate used which was L-galactose in this Example. The amounts of L-galactono-1,4-lactone plus L-galactonic acid and AsA are summarized in Table 3.

Table 3

Strain	IPTG	HPLC	
		L-GaL+L-GaA (mM)	AsA (mg/L)
JM109 /pTrcMalE-EnzB	+	6.2	2.7
	-	3.9	1.6
JM109	+	nd	nd
	-	nd	nd

L-GaL: L-galactono-1,4-lactone; L-GaA: L-galactonic acid; nd: not detected

Example 4: Production of AsA from L-galactono-1,4-lactone/L-galactonic acid by *Escherichia coli* JM109 carrying the Enzyme B gene

The experiment was performed as described in Example 1, except of the substrate used which was L-galactono-1,4-lactone in this Example. The amount of AsA is summarized in Table 4.

Table 4

Strain	IPTG	HPLC
		AsA (mg/L)
JM109 /pTrcMalE-EnzB	+	4.7
	-	3.7
JM109	+	nd
	-	nd

nd: not detected

Claims

1. The use of Enzyme B of *G. oxydans* DSM 4025, as disclosed in EP 832,974, in a process for producing L-ascorbic acid from L-gulose, L-galactose, L-idose or L-talose, or from L-gulono-1,4-lactone (and its acid form, L-gulonic acid) and from L-galactono-1,4-lactone (and its acid form, L-galactonic acid).

2. The use of Enzyme B of *G. oxydans* DSM 4025, as disclosed in EP 832,974, in a process for producing L-gulono-1,4-lactone or L-galactono-1,4-lactone, or their acid forms L-gulonic acid or L-galactonic acid from L-gulose or L-galactose, respectively.

3. The use of Enzyme B of *G. oxydans* DSM 4025, as disclosed in EP 832,974, in a process for producing L-ascorbic acid from L-gulono-1,4-lactone or L-galactono-1,4-lactone, or their acid forms L-gulonic acid or L-galactonic acid.

4. A process for (a) producing
(i) L-ascorbic acid from L-gulose or L-galactose by an enzyme which comprises contacting L-gulose or L-galactose with an enzyme having an amino acid sequence of SEQ ID NO:2 or an amino acid sequence that is 90% identical thereto, or a portion thereof, with the activity to produce L-ascorbic acid from L-gulose and L-galactose or a functional equivalent thereof, in a reaction mixture,
(ii) L-gulono-1,4-lactone from L-gulose, or L-galactono-1,4-lactone from L-galactose by an enzyme which comprises contacting L-gulose or L-galactose with an enzyme having an amino acid sequence of SEQ ID NO:2 or an amino acid sequence that is 90% identical thereto, or a portion thereof, with the activity to produce L-gulono-1,4-lactone from L-gulose and L-galactono-1,4-lactone from L-galactose, or a functional equivalent thereof, in a reaction mixture, or
(iii) L-ascorbic acid from L-gulono-1,4-lactone or from L-galactono-1,4-lactone by an enzyme which comprises contacting L-gulono-1,4-lactone or from L-galactono-1,4-lactone with the enzyme having an amino acid sequence of SEQ IDNO:2 or an amino acid sequence that is 90% identical thereto, or a portion thereof, with the activity to produce L-ascorbic acid from L-gulono-1,4-lactone and from L-galactono-1,4-lactone, or a functional equivalent thereof, in a reaction mixture,
and (b) isolating L-ascorbic acid, L-gulono-1,4-lactone or L-galactono-1,4-lactone from the reaction mixture.

5. The process according to any one of claim 1, 2, 3 or 4, wherein the contact of the enzyme and the substrate is conducted at a pH in the range of from 1 to 9, preferably from

2 to 8, at a temperature in the range of from 13°C to 45°C, preferably from 18°C to 42°C, for 1 to 120 hours.

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SEQUENCE LISTING

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Tyr Asp Ile Met Ala Val Asp Gln Glu Phe Ser Ala Leu Asp Val Tyr
        435                 440                 445

aac acc agc gcg acc gca aaa ctc gcg ccg ggc ttt gaa aat atg ggc     1392
Asn Thr Ser Ala Thr Ala Lys Leu Ala Pro Gly Phe Glu Asn Met Gly
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cgc atc gac gcg att gat atc agc acc ggg cgc acc ttg tgg tcg gcg     1440
Arg Ile Asp Ala Ile Asp Ile Ser Thr Gly Arg Thr Leu Trp Ser Ala
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gag cgc cct gcg gcg aac tac tcg ccc gtt ttg tcg acg gca ggc ggt     1488
Glu Arg Pro Ala Ala Asn Tyr Ser Pro Val Leu Ser Thr Ala Gly Gly
                485                 490                 495

gtg gtg ttc aac ggc ggg acc gac cgc tat ttc cgt gcc ctc agc cag     1536
Val Val Phe Asn Gly Gly Thr Asp Arg Tyr Phe Arg Ala Leu Ser Gln
            500                 505                 510

gaa acc ggc gag act ttg tgg cag gcc cgt ctt gcg acg gtc gcg acg     1584
Glu Thr Gly Glu Thr Leu Trp Gln Ala Arg Leu Ala Thr Val Ala Thr
        515                 520                 525

ggg cag gcg atc agc tac gag ttg gac ggc gtg caa tat atc gcc atc     1632
Gly Gln Ala Ile Ser Tyr Glu Leu Asp Gly Val Gln Tyr Ile Ala Ile
    530                 535                 540

ggt gcg ggc ggt ctg acc tat ggc acg caa ttg aac gcg ccg ctg gcc     1680
Gly Ala Gly Gly Leu Thr Tyr Gly Thr Gln Leu Asn Ala Pro Leu Ala
545                 550                 555                 560

gag gca atc gat tcg acc tcg gtc ggt aat gcg atc tat gtc ttt gca     1728
Glu Ala Ile Asp Ser Thr Ser Val Gly Asn Ala Ile Tyr Val Phe Ala
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ctg ccg cag taa                                                    1740
Leu Pro Gln
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<213> Gluconobacter oxydans
<400> 2

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Thr Ala Pro Ala Ala Phe Ala Gln Val Thr Pro Ile Thr Asp Glu Leu
            20                  25                  30
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Leu Ala Asn Pro Pro Ala Gly Glu Trp Ile Asn Tyr Gly Arg Asn Gln
35 40 45

Glu Asn Tyr Arg His Ser Pro Leu Thr Gln Ile Thr Ala Asp Asn Val
50 55 60

Gly Gln Leu Gln Leu Val Trp Ala Arg Gly Met Glu Ala Gly Ala Val
65 70 75 80

Gln Val Thr Pro Met Ile His Asp Gly Val Met Tyr Leu Ala Asn Pro
85 90 95

Gly Asp Val Ile Gln Ala Leu Asp Ala Gln Thr Gly Asp Leu Ile Trp
100 105 110

Glu His Arg Arg Gln Leu Pro Ala Val Ala Thr Leu Asn Ala Gln Gly
115 120 125

Asp Arg Lys Arg Gly Val Ala Leu Tyr Gly Thr Ser Leu Tyr Phe Ser
130 135 140

Ser Trp Asp Asn His Leu Ile Ala Leu Asp Met Glu Thr Gly Gln Val
145 150 155 160

Val Phe Asp Val Glu Arg Gly Ser Gly Glu Asp Gly Leu Thr Ser Asn
165 170 175

Thr Thr Gly Pro Ile Val Ala Asn Gly Val Ile Val Ala Gly Ser Thr
180 185 190

Cys Gln Tyr Ser Pro Tyr Gly Cys Phe Ile Ser Gly His Asp Ser Ala
195 200 205

Thr Gly Glu Glu Leu Trp Arg Asn His Phe Ile Pro Gln Pro Gly Glu
210 215 220

Glu Gly Asp Glu Thr Trp Gly Asn Asp Phe Glu Ala Arg Trp Met Thr
225 230 235 240

Gly Val Trp Gly Gln Ile Thr Tyr Asp Pro Val Thr Asn Leu Val Phe
245 250 255

Tyr Gly Ser Thr Gly Val Gly Pro Ala Ser Glu Thr Gln Arg Gly Thr
260 265 270

Pro Gly Gly Thr Leu Tyr Gly Thr Asn Thr Arg Phe Ala Val Arg Pro
275 280 285

Asp Thr Gly Glu Ile Val Trp Arg His Gln Thr Leu Pro Arg Asp Asn
290 295 300

Trp Asp Gln Glu Cys Thr Phe Glu Met Met Val Ala Asn Val Asp Val
305 310 315 320

Gln Pro Ser Ala Glu Met Glu Gly Leu Arg Ala Ile Asn Pro Asn Ala
325 330 335

Ala Thr Gly Glu Arg Arg Val Leu Thr Gly Ala Pro Cys Lys Thr Gly
340 345 350

Thr Met Trp Ser Phe Asp Ala Ala Ser Gly Glu Phe Leu Trp Ala Arg
355 360 365

Asp Thr Asn Tyr Thr Asn Met Ile Ala Ser Ile Asp Glu Thr Gly Leu
370 375 380

Val Thr Val Asn Glu Asp Ala Val Leu Lys Glu Leu Asp Val Glu Tyr
385 390 395 400

Asp Val Cys Pro Thr Phe Leu Gly Gly Arg Asp Trp Ser Ser Ala Ala
405 410 415

Leu Asn Pro Asp Thr Gly Ile Tyr Phe Leu Pro Leu Asn Asn Ala Cys
420 425 430

Tyr Asp Ile Met Ala Val Asp Gln Glu Phe Ser Ala Leu Asp Val Tyr
435 440 445

Asn Thr Ser Ala Thr Ala Lys Leu Ala Pro Gly Phe Glu Asn Met Gly
450 455 460

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Arg Ile Asp Ala Ile Asp Ile Ser Thr Gly Arg Thr Leu Trp Ser Ala
465                 470                 475                 480

Glu Arg Pro Ala Ala Asn Tyr Ser Pro Val Leu Ser Thr Ala Gly Gly
                485                 490                 495

Val Val Phe Asn Gly Gly Thr Asp Arg Tyr Phe Arg Ala Leu Ser Gln
            500                 505                 510

Glu Thr Gly Glu Thr Leu Trp Gln Ala Arg Leu Ala Thr Val Ala Thr
        515                 520                 525

Gly Gln Ala Ile Ser Tyr Glu Leu Asp Gly Val Gln Tyr Ile Ala Ile
    530                 535                 540

Gly Ala Gly Gly Leu Thr Tyr Gly Thr Gln Leu Asn Ala Pro Leu Ala
545                 550                 555                 560

Glu Ala Ile Asp Ser Thr Ser Val Gly Asn Ala Ile Tyr Val Phe Ala
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Leu Pro Gln
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